DNA methylation profiling of esophageal adenocarcinoma using Methylation Ligation-dependent Macroarray (MLM)

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Abstract

Most types of cancer cells are characterized by aberrant methylation of promoter genes. In this study, we described a rapid, reproducible, and relatively inexpensive approach allowing the detection of multiple human methylated promoter genes from many tissue samples, without the need of bisulfite conversion. The Methylation Ligation-dependent Macroarray (MLM), an array-based analysis, was designed in order to measure methylation levels of 58 genes previously described as putative biomarkers of cancer. The performance of the design was proven by screening the methylation profile of DNA from esophageal cell lines, as well as microdissected formalin-fixed and paraffin-embedded (FFPE) tissues from esophageal adenocarcinoma (EAC). Using the MLM approach, we identified 32 (55%) hypermethylated promoters in EAC, and not or rarely methylated in normal tissues. Among them, 21 promoters were found aberrantly methylated in more than half of tumors. Moreover, seven of them (*ADAMTS18*, *APC*, *DKK2*, *FOXL2*, *GPX3*, *TIMP3* and *WIF1*) were found aberrantly methylated in all or almost all the tumor samples, suggesting an important role for these genes in EAC. In addition, dysregulation of the Wnt pathway with hypermethylation of several Wnt antagonist genes was frequently observed. MLM revealed a homogeneous pattern of methylation for a majority of tumors which were associated with an advanced stage at presentation and a poor prognosis. Interestingly, the few tumors presenting less methylation changes had a lower pathological stage. In conclusion, this study demonstrated the feasibility and accuracy of MLM for DNA methylation profiling of FFPE tissue samples.

Keywords: DNA methylation; profiling; esophageal adenocarcinoma; FFPE

1. Introduction

In mammals, DNA methylation occurs almost exclusively at the carbon-5 position of cytosine residues within CpG dinucleotides. These dinucleotides are mainly clustered in regions named CpG islands usually surrounding gene promoters or regulatory regions [1]. Disruption of DNA methylation profiles is a well-characterized epigenetic hallmark for various pathologies including cancer [2,3]. Most types of cancer cells are characterized by a global loss of DNA methylation that contributes to genomic instability, concomitantly to hypermethylation of specific promoters which leads to transcriptional inactivation of many genes including tumor suppressor genes [3]. Because these alterations can occur very early, their detection is of importance for diagnosis, prognosis and treatment of cancer.

A wide range of options exists to study DNA methylation. Among these, analysis of bisulfite-treated genomic DNA is a standard technique based on the ability of sodium bisulfite to deaminate cytosine residues into uracils, whereas the methylated cytosines are resistant to this modification and thus remain unchanged. The original technique, described in 1992, included DNA sequencing of PCR products [4]. Today, additional methods including bisulfite-DNA as a starting point for methylation analysis are commonly used, such as methylation specific PCR (MSP), single strand conformation analysis (MS-SSCA) or pyrosequencing [5]. On another hand, technologies as high-throughput sequencing from bisulfite-DNA can be used for genomic screening to increase our knowledge on regions potentially methylated. However, these technologies are still expensive, complex to analyze and thus, not yet suitable for routine testing [6].

An alternative to DNA bisulfite-treatment is to identify methylated DNA regions by digestion with methylation-sensitive restriction enzymes followed by PCR or quantitative PCR analysis of the region(s) of interest [7]. The recently developed Methylation-Specific Multiplex

Ligation-dependent Probe Amplification (MS-MLPA) can detect changes in CpG methylation up to 40 chromosomal sequences in a simple reaction without bisulfite conversion [8,9]. The various amplicons obtained after PCR amplification are discriminated on the basis of size. Although robust, MS-MLPA is not flexible because new MLPA probes can hardly be added in the reaction mix. Therefore, there is still a need to develop a simple method for the simultaneous analysis of methylation changes in a large number of genes.

In this paper, we describe the successful development of such a method for DNA methylation profiling that we named Methylation Ligation-dependent Macroarray (MLM). Formalin-fixed and paraffin embedded tissue (FFPE) is the standard in evaluation of specimen and diagnosis in routine and MLM can be easily applied to FFPE tissue samples. Using MLM, we analyzed CpG sites in multiple promoter regions described as candidate DNA methylation biomarkers in esophageal adenocarcinoma (EAC) originated from Barrett's esophagus (BE), a pre-malignant condition where the squamous epithelium of the tubular esophagus is replaced by specialized intestinal-type columnar epithelium [10].

2. Material and Methods

2.1. Cell lines

OE-19, OE-33 and TE-7 human esophageal tumor cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. OE-19 and OE-33 were derived from EAC, and TE-7 from a esophageal squamous cell carcinoma.

2.2. Tissue samples, Microdissection and DNA extraction

Tissue samples were collected between 1983 and 2001 from esophagectomy sections on patients with an EAC [11]. All the samples - including 4 normal esophageal epithelium, 4

normal gastric epithelium, and 26 EAC samples - were formalin-fixed and paraffin-embedded (FFPE). Manual microdissection of FFPE tissue sections, in order to enrich the samples to at least 70% of tumor cells, and DNA extraction were performed as previously described [12]. According to the TNM staging classification, the 26 EAC samples were: 5(19%)T1N0M0, 1 (4%) T1N1M0, 1 (4%) T2N0M0, 3(11%) T3N0M0, 12 (46%) T3N1M0, 2 (8%) T3N1M1 and 2 (8%) T4N1M1.

2.3. MS-SSCA and bisulfite pyrosequencing

Bisulfite processing of genomic DNA was performed using the EpiTectBisulfite Kit according to the manufacturer's protocol (Qiagen). Modified-bisulfite DNA was PCR amplified and submitted to methylation-sensitive single-strand conformation analysis (SSCA), as previously described [13]. Pyrosequencing was performed on the PyroMark Q24 according to the manufacturer's recommendations (Qiagen).

2.4. MLM: primer design

For each of the 58 gene promoter region of interest, two primers (A and B) were designed to be contiguous so they can be ligated to each other when hybridized to their target sequence. Each couple of primers contained at least one *CfoI* restriction site (GCGC), except the two ligation controls (CTRL-LIG1 and CTRL-LIG2) that are restriction site-free. In addition, A primers contained the same 5'-GGGTTCCCTAAGGGTTGGAtag (MLM-F) while B primers contained the 3'-TCTAGATTGGATCTTGCTGGCACtag (MLM-R), these tags will be used in the ultimate PCR step. B-primers also contained a 5'-phosphate. The primer and probe sequences are available on request.

2.5. MLM: sample preparation

Genomic DNA extracted from cell lines was sonicated to generate 300-500 bp fragments. Sonication was not needed for DNA extracted from FFPE tissues. Forty ng of DNA were

incubated with the mix of primers (0.75 fmoles of each) and 1.5 µl of MLPA buffer (MRC Holland) in 8 µl final volume. DNA was denatured at 98°C for 10min and then kept at 55°C for 16h for probe hybridization. After addition of 3µl ligase buffer A (MRC Holland) and 11 µl water, sample were equally divided into two PCR tubes. Ligase buffer B (1.5 µl, MRC Holland), water (8.5 µl) and *CfoI* enzyme (10U, Roche) were added in one tube (assay), whereas the second tube was prepared the same way without *CfoI* (control). DNA digestion was performed at 37°C for 5h, followed by 58°C for 5min for enzyme inactivation. A mix of 5 µl, containing *Taq* ligase, ligase buffers A and B (0.6 µl, 0.375 µl and 0.375 µl, respectively), was added in all tubes. Samples were incubated 30min at 54°C for ligation, followed by 5min at 98°C for ligase inactivation. A PCR reaction with the MLM-F-biotin and MLM-R primers was then performed on all samples for 35 cycles at 60°C. Size of amplicons (85-110 bp) was checked on agarose gel.

2.6. MLM: macroarray design and preparation

Array probes, with a 5'-amino function, were able to hybridize to their corresponding MLM products. A nylon membrane (Biodyne® C, 0.45 µm, Life Sciences) was activated with 16% EDAC solution and placed in a Miniblotter®45 (Immunetics). Each channel was loaded with 100 µM of one probe diluted in NaHCO₃ 0.5M, pH8.4. The membrane was inactivated with 10 mM NaOH at room temperature for 10min, and washed twice with 2X SSPE/0.1% SDS at 70°C for 10min. Finally, the membrane was rinsed with 20 mM EDTA and stored at 4°C into a fresh EDTA solution.

2.7. MLM: macroarray hybridization, image acquisition and data processing

The spotted membrane was placed in the Miniblotter®45 and channels are filled with 2X SSPE/0.1% SDS for pre-warming, 1h at 58°C. Ten microliters of biotin-labeled PCR products mixed with 40 µl of blue dye (Ficoll, Xylene cyanol and Bromophenol blue) were denatured

at 98°C for 10min and loaded onto the membrane after addition of 150 µl of 8X SSPE/0.1% SDS. After 1h hybridization at 58°C, the membrane was washed with 2X SSPE/0.1% SDS twice 15min at 58°C, twice 1min at 58°C, and twice at room temperature. Then, the membrane was incubated for 30min with 2X SSPE/0.5% SDS containing a 1:10000 dilution of Streptavidin-AP (Roche). After washing with 2X SSPE/0.5% SDS, 2X SSPE and detection buffer, the membrane was incubated with CDP-Star (Roche) to reveal hybridization.

Chemiluminescence was acquired by the Luminescent Image Analyzer “LAS-1000 plus” (Fujifilm). The array images were converted into spot intensity values. All the samples for which the intensity values of CTRL-LIG1 and CTRL-LIG2 (restriction site-free targets) were at least five times above the background were retained for analysis. Then β -values were calculated as follow: $\beta = (\text{intensity value in assay} / \text{intensity in control})$ divided by the mean β -value of CTRL-LIG1 and CTRL-LIG2. Finally, $\beta \leq 0$ were replaced by 0.01 while $\beta \geq 1$ were replaced by 0.99.

2.8. DNA methylation clustering

Data were clustered in a hierarchical tree using the software Cluster and Treeview [14], available at <http://rana.lbl.gov/EisenSoftware>. Before launching the "complete clustering" procedure, the β -values were converted in M-values in which $M = \log_2[\beta / (1 - \beta)]$.

2.9. Statistics

To assess the variability, β values were independently established for each replicate. The β values that were detected as outliers for probe, i.e. Mean ± 2 *Standard Deviation, were counted. Then the number of these outliers divided by the total number of spots was used as a measure of the assay's variability. Coefficient of variation (CV) corresponds to the SD divided by the number of replicates. The observed R values were computed using Vassarstat tool (<http://faculty.vassar.edu/lowry/VassarStats.html>).

3. Results

3.1. MLM design

The MLM has been designed to be rapid, reproducible, and simple of use as bisulfite conversion is not required (Fig. 1). For cell lines, genomic DNA fragmentation was performed by sonication to obtain homogenized DNA sizes (1st step). After DNA denaturation and primer hybridization on the targeted regions of interest (2nd step), the DNA was cleaved at GCGC sites by the methylation sensitive restriction enzyme *CfoI* if unmethylated, whereas the methylated DNA remained intact. Primers hybridized on this latter are then ligated to form a double-strand matrix (3rd step). To avoid misinterpretation, half of each sample is incubated as its counterpart but without the restriction enzyme *CfoI*, leading to a positive signal (control). The ligated DNA fragments are amplified by PCR using universal primers on the extremities of each targeted sequence (4th step). All targeted sequences are of small size (85-110 bp) and similar base composition, allowing a multiplex exponential amplification at a unique hybridization temperature without bias. For each targeted sequence, one of the PCR primers is tagged with a biotin molecule. All probes corresponding to the targeted regions are captured on a nylon membrane using a multichannel support. Negative and positive controls are included to enable a correct reading of the results and to provide a semi-quantitative analysis of DNA methylation. After hybridization of the PCR products, amplicons are detected by chemiluminescence (5th step). A high-resolution image of the chemiluminescent array is done using a CCD camera. The image is further analyzed visually or the spot intensities quantified using array analysis software.

3.2. MLM reproducibility

MLM assays were performed as described in Fig. 1 using genomic DNA from OE-19. Eleven replicates were analyzed. The restriction-free targets (CTRL-LIG1 and CTRL-LIG2) and some genes (*FBP1*, *APC*, *WIF1*, *BORIS*, *TIMP3* and *hTERT*) produced a high signal in both control (-) and assay (+) column, while other genes (*CDKN2A*, *CDH1*, *RASSF1A* and *MLH1*) presented a high signal only in the control, providing evidence that primer annealing, *CfoI* digestion and array hybridization were efficient (Fig. 2A). A β -value of 0 means the analyzed CpGsite is completely unmethylated and a β -value of 1 means fully methylated. Only 5% of signals, 34 spots over 638 (11 replicates for 58 genes), revealed a relevant β -value deviation of 0.15-0.30 (min-max). These results indicated that the MLM technical procedure and signal processing were highly robust and remained consistent across independent MLM reactions.

3.3. MLM accuracy

We next evaluated if the MLM technique is able to accurately determine DNA methylation level. Data obtained for OE-19 suggested that the CpG screened for *CDH1*, *CDKN2A*, *MLH1* and *RASSF1A* regions were hypomethylated ($0.04 \leq \text{mean } \beta\text{-value} \leq 0.28$), and those of *APC*, *BORIS*, *FBP1* and *WIF1* regions were strongly methylated ($0.80 \leq \text{mean } \beta\text{-value} \leq 0.96$). *hTERT* harbored an intermediate level of methylation (mean β -value of 0.54) (Fig. 2A). Similar levels of methylation were observed by MS-SSCA or bisulfite pyrosequencing (Fig. 2B). The MLM data were thus in perfect agreement with those obtained with bisulfite-based methods.

3.4. MLM testing semi-quantification

DNA mixtures from OE-19 and TE-7 were tested by MLM. A gradual decrease or increase of the signal was observed for the 5 promoter regions investigated in adequacy with the

predictable degree of methylation (Fig. 2C). These results show the ability of MLM to determine the methylation status of heterogeneous samples. Nevertheless, the analytical sensitivity implies a minimum of 25% methylation difference between two DNA populations in a sample for accurate methylation analysis.

3.5. Methylation profiling of esophageal adenocarcinoma FFPE specimens

All the above data indicated that the MLM performance depends on the methylation difference between normal and tumor areas. This finding highlights the importance of tumor enrichment by microdissection in order to increase the sensitivity and accuracy of methylation profiling. Therefore, in our series of 26 EAC, manual microdissection has been performed on FFPE tissues to reach a minimum of 70% of tumor cells.

In the 26 EAC samples investigated in this study (Fig. 3), the methylation levels of 10 out of the 58 promoter regions have been previously determined by MS-SSCA after bisulfite modification [11,15]. Using MLM, the level of methylation was considered low for hypomethylation (β -value <0.4 , white color), intermediate (β -value ≥ 0.2 and ≤ 0.7 , gray color) and high for hypermethylation (β -value >0.7 , black color). A difference in the level of methylation (low, intermediate or high) was noticed for only 8 among the 260 analyses, which indicates a concordance $\geq 97\%$ between MLM and MS-SSCA. MLM in EAC samples revealed an aberrant hypermethylation in 55% (32/58) of promoter genes, these genes were usually found hypomethylated in normal esophagus and gastric tissues.

Data were then clustered in a hierarchical tree (Fig. 4). The first cluster contained all the normal tissue samples and T7 cell line. In this cluster, only 8(16%) genes were found hypermethylated (*ADAMTS12*, *AXIN2*, *BORIS*, *CDH13*, *CDX1*, *SEPT9*, *SPARC*, *SFRP1* and *TAC1*), however this hypermethylation was also noticed in almost all the second cluster samples. This second cluster contained all the EAC, 26 tumors and 2 cell lines. Promoter

hypermethylation was observed for 21 (36%) genes in more than half of tumors, whereas normal tissue samples were hypomethylated. Interestingly, for 7 of these genes (*ADAMTS18*, *APC*, *DKK2*, *FOXL2*, *GPX3*, *TIMP3* and *WIF1*), hypermethylation was found in all or almost all the tumor tissues, suggesting an important role for these genes in EAC. In addition, 11 (19%) promoters (*DKK1*, *DKK3*, *GATA5*, *MLH1*, *PAX6*, *PTGS2*, *RASSF2A*, *TP73*, *TWIST1*, *WNT2* and *WRN*) were found aberrantly methylated in only a subset of tumors. An absence of hypermethylation in both normal and neoplastic tissues was only noticed in a few genes, such as *SMAD4*, *SOCS1* and *WNT4*. The second cluster is represented by 4 subclusters. The first 2 contained 8 EAC with less hypermethylated genes, and all but one of the T1 or T2 (6/7) tumors were in these subclusters. Similarly, with an overall higher number of methylated genes, all but one of the 19 EAC of groups 3 and 4 were T3 or T4 tumors.

4. Discussion

A new technique allowing the detection and semi-quantification of multiple methylated gene sequences in a simple reaction was presented. This approach, termed MLM (for Methylation Ligation-dependent Macroarray), was developed in order to be rapid, easy to perform, reproducible, and relatively inexpensive. The choice of the CpG site for each analyzed gene was based on literature data and evidences of robust methylated sites. Most CpG were located in CpG islands and designing the primers is important to ensure an unbiased hybridization and multiplex exponential amplification. In contrast to MLPA currently in use [10], primers could be adapted very easily by respecting some state-of-the-art rules. Therefore, genes that may be specific of certain tumor subtype could be rapidly screened by preparing a tumor specific cocktail of primers. The MLM can be performed in a laboratory without special equipment,

making it very convenient. Because there is no need to bisulfite conversion, a MLM analysis takes about 2 days, including many controls to ensure the reproducibility of the assay. Furthermore, the membrane can be stripped and re-used at least 10 times, reducing the cost of the analysis. The technique used a small amount of DNA, which has been a limitation in other assays, making it suitable for the analysis of FFPE samples in research and routine diagnostic. While applied to human samples, the MLM showed its robustness to identify methylated CpG. Using FFPE tissues, MLM data were a like those obtained using bisulfite-based techniques, which are widely used in the detection of DNA methylation[13,16]. Indeed, an excellent concordance (> 97%) was found between bisulfite-PCR analysis and MLM in our FFPE samples. PCR analysis after bisulfite conversion of DNA from FFPE samples is often challenging because bisulfite conversion results in extensive degradation of DNA, leading to a decrease in the number of interpreted cases. Therefore, using MLM, reliable methylation profiles, can be easily obtained from FFPE samples, without the need of bisulfite conversion. Data interpretation after MLM is fast, easy and achievable in all the collected FFPE tissues making feasible the application of this technique to routine specimens. One limitation of MLM could be the relatively small number of studied genes with respect to other profiling techniques, such as methylation microarray [17]. Nevertheless, using MLM, a set of 30-50 genes implicated in key neoplastic pathways can be selected, which is largely sufficient for the great majority of clinical applications.

In the present study, we characterized the methylation profile of 58 promoter genes in EAC. We identified 32 (55%) promoters with aberrant hypermethylation, which were almost always unmethylated in normal esophagus and gastric tissues. Among them, 21 genes (36%) were found hypermethylated in more than half of tumors and even for seven of them promoter hypermethylation was found in almost all the tumors tissues investigated suggesting an important role for these genes in EAC carcinogenesis. Among the most common

hypermethylated genes found in our series of EAC, most of them have been reported previously to be hypermethylated in EAC as well as in other cancers, such as *APC*, *hTERT*, *SFRPs* and *TIMP3*[11], *CALCA*, *ESR1*, *MGMT* and *MYOD1*[18], and *RUNX3* and *TMEFF2* [19]. A subset of these abnormally methylated tumor suppressor genes (*APC*, *TIMP3*, *hTERT* and *WIF1*), play an important role in the pathogenesis of EAC as already reported in previous studies and might be useful prognostic markers as they appear to precede and thus predict the progression of BE (Barrett's esophagus) to EAC [11,15]. Few others hypermethylated genes, including *ADAMTS18*, *ALX4*, *DKK2*, *FOXL2* and *RSPO1*, were already reported in cancers but not in EAC [20-22]. In our series of EAC, high frequency of promoter methylation ($\geq 80\%$ of tumors) was observed for several Wnt repressors, including *APC*, *DKK1*, *DKK2*, *SFRP1*, *SFRP2*, *SFRP4* and *WIF1*. The Wnt/ β -catenin signaling pathway is involved in a wide-range of biological processes, including embryogenesis and tumor formation [23]. Dysregulation of the Wnt pathway is common in many type of cancers, such as colon, breast, lung, prostate and esophagus [24,25]. In most cancers, activation of the Wnt pathway is mainly the consequence of the transcriptional repression by promoter methylation of negative regulators of Wnt pathway [26].

The results of this study show that our series of EAC has a relatively homogeneous methylation pattern differently to what reported for other neoplasia. Similarly, using MLM in colon, skin and ovarian cancer, we observed heterogeneous methylation pattern (unpublished data), which could be associated to different clinical outcome. In fact, almost all the examined EAC were characterized by a similar advanced stage at presentation and a poor survival. Interestingly, the few tumors presenting less hypermethylated genes showed lower pathological stage. A homogeneous methylation pattern was also observed in EAC using a large-scale array-based DNA methylation analysis and not a candidate-gene approach[17].

In summary, MLM could provide a new opportunity to perform DNA methylation profiling in all clinical samples that have been stored as FFPE tissue blocks. MLM is a rapid, reproducible, and relatively inexpensive method that could be used to determine DNA methylation profiling into clinical practice to provide personalized treatment.

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Competing financial interests

The authors have no competing financial interests.

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Legends to Figures

Fig. 1. Methylation Ligation-dependent Macroarray (MLM) method scheme. A MLM assay is performed in 5 steps. The 1st step is not needed for FFPE tissue samples. For each sample, a control without *CfoI* digestion is included.

Fig. 2. MLM assay: reproducibility, accuracy and semi-quantification of methylation levels. A) DNA from OE-19 was analyzed by MLM in four experimental replicates. Each row represents a gene and each double column a replicate. For each sample (+), a control without digestion (-) and 2 controls for ligation (CTRL-LIG1 and CTRL-LIG2) are included. Methylation levels were calculated as β -values ranging from 0 for unmethylated to 1 for fully methylated CpG sites. B) Comparison of promoter methylation levels in OE-19 determined by MLM and bisulfite-based methods (MS-SSCA and bisulfite pyrosequencing). C) TE-7 DNA was added in increasing amounts to OE-19 DNA to create a DNA dilution series. Detection of methylation changes in DNA mixtures is highlight for 5 genes: *APC*, *RASSF1A*, *hTERT*, *WIF1* and *MLH1*.

Fig. 3. MLM in 26 FFPE EAC samples. Methylation profiling of 58 promoter genes were performed in cell lines (TE-7, OE-19, OE-33), in 8 normal esophagus (NE) and gastric tissues (NG), and in 26 microdissected FFPE EAC tissues (T). Each row represents a gene and each column a sample. White boxes indicate hypomethylation (β -value <0.4), grey boxes partial methylation (β -value ≥ 0.2 and ≤ 0.7), and black boxes hypermethylation (β -value >0.7).

Fig. 4. Hierarchical clustering analysis of 58 differentially methylated gene promoters in EAC after MLM analysis. Normal esophageal (NE) and gastric (NG) tissues are indicated in

red. EAC (T) are in black. Cell lines are in violet. The methylation levels (β -values) varies from 0 (0% methylation, green) to 1 (100% methylation, red).

Figure 1

B

Gene	MLM	MS-SSCA	Bisulfite Pyrosequencing
APC	85	Methyl.	ND
BORIS	80	ND	94
CDH1	26	Unmethyl.	ND
FBP1	96	ND	92
hTERT	54	ND	49
MLH1	4	Unmethyl.	8
CDKN2A	28	Unmethyl.	ND
RASSF1A	14	ND	10
WIF1	82	Methyl.	ND

Figure 2

Figure 3

Figure 4